Communications to the Editor

Incorporation of Nonnatural Amino Acids into Streptavidin through *In Vitro* Frame-Shift Suppression

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Incorporation of nonnatural amino acids that carry a variety of functional side groups into proteins is a versatile technique for introducing artificial functions into proteins.[1,2] *In vitro* suppression of amber termination codon has been used for this purpose,[3,4] although the suppression competes with termination of protein synthesis and only two of three termination codons are available. Recently, we reported incorporation of photofunctional nonnatural amino acids into a polypeptide through *Escherichia coli in vitro* protein biosynthesis.[5] In the latter case, AGG was chosen as a codon for the nonnatural amino acids, since AGG is rarely used in *E. coli*.[6] The use of the minor codon extends the possibility of the technique to incorporate several different amino acids into proteins.

In this paper, we propose novel codon−anticodon pairs for the incorporation of nonnatural amino acids into proteins through frame-shift suppression. Combinations of four-base codons AGGN and the corresponding anticodons NCCU were chosen to examine the possibility of the frame-shift suppression. Since the translation of AGG to arginine is inefficient, the AGGN will be preferentially read by the $tRNA_{NCCU}$ that are added to the *in vitro* system, rather than by the endogenous $tRNA_{CCU}$.[7]

Streptavidin was used as a target protein. A synthetic gene for streptavidin was purchased from R&D Systems Europe and site-directed mutagenesis was performed by PCR technique to replace TAT(Tyr83) by four-base codons AGGN. The mutant gene was fused with T7-tag at the N-terminal under T7 promoter and with His-tag at the C-terminal. The mRNA was synthesized *in vitro* by T7 RNA polymerase.

The sequence of the mutation region was designed as shown in Scheme 1. When the frame-shift does not occur, a termination codon UAA appears after UCG(Ser) and the protein synthesis is truncated. However, if the frame is shifted by $tRNA_{NCCU}$s charged with nonnatural amino acids, the protein synthesis continues down to the C-terminal. As the result, the full-length streptavidin sequence must contain nonnatural amino acid at the mutation site.

The $tRNA_{NCCU}$s were designed according to the sequence of yeast $tRNA^{Phe}$.[8] The template DNA encoding $tRNA_{ACCU}$(-CA) under T7 promoter was chemically synthesized, and the templates of other tRNA(-CA)s were generated by site-directed mutagenesis. The aminoacyl tRNAs were prepared by a ligation of chemically synthesized aminoacyl dinucleotide to T7-transcribed $tRNA_{NCCU}$(-CA)s with T4 RNA ligase.[9] *In vitro* protein synthesis was performed in *E. coli* S-30 system.[10] The western blot analysis was performed with anti-T7 tag antibody (Novagen) and alkaliphosphatase-labeled anti-mouse IgG.

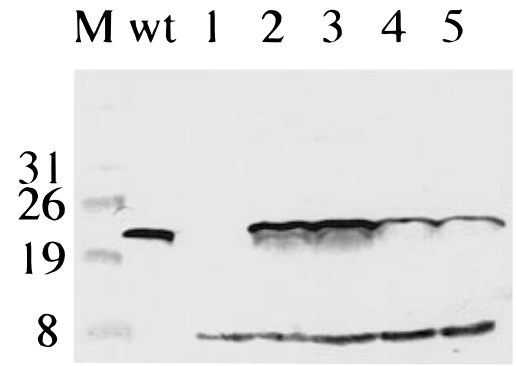

Figure 1. Western blot analysis of *in vitro* translation. The *in vitro* reaction mixtures (2 μL) were applied to 15% SDS-PAGE and transferred to nitrocellulose membrane, followed by incubation with anti-T7 tag antibody. The bands were visualized with alkaliphosphatase-labeled anti-mouse IgG and NBT/BCIP. The reaction contains the following: lane 1, nonacylated $tRNA_{ACCU}$; lane 2, nitrophenylalanyl $tRNA_{ACCU}$; lane 3, 2-naphthylalanyl $tRNA_{ACCU}$; lane 4, (*p*-(phenylazo)-phenyl)alanyl $tRNA_{ACCU}$; lane 5, 2-anthrylalanyl $tRNA_{ACCU}$. Lane M and lane wt contain prestained molecular weight standards and the *in vitro* reaction (0.4 μL) of wild type mRNA, respectively. The bands of 20 kDa correspond to T7- and His-tagged streptavidin monomer.

Scheme 1

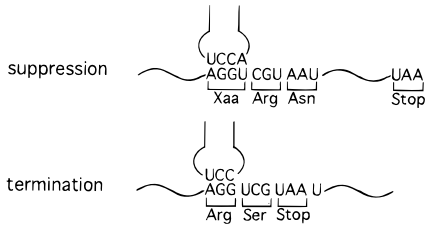

Figure 1 shows results of the translation of the frame-shift mutant mRNA containing AGGU. When nonacylated $tRNA_{ACCU}$ was added, no streptavidin was synthesized. This result suggests that the *in vitro* system does not suppress the frame-shift mutation and that the $tRNA_{ACCU}$ is not recognized by any endogenous aminoacyl tRNA synthetases. However, in the presence of tRNA chemically acylated with nitrophenylalanine, streptavidin was successfully synthesized. This is interpreted in terms of the suppression of the frame-shift mutation by nitrophenylalanyl $tRNA_{ACCU}$. Other nonnatural amino acids carrying 2-naphthyl, *p*-(phenylazo)phenyl, and

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(2) Baldini, G.; Martoglio, A.; Zugliani, C.; Brunner, J. *Biochemistry* **1988**, *27*, 7951−7959.
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(4) Bain, J. D.; Glabe, C. G.; Dix, T. A.; Chamberlin, A. R.; Diala, E. S. *J. Am. Chem. Soc.* **1989**, *111*, 8013−8014.
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(6) Wada, K.; Aota, S.; Tsuchiya, R.; Ishibashi, F.; Gojobori, T.; Ikemura, T. *Nucleic Acids Res.* **1990**, *18*, 2367−2411.
(7) Ma, C.; Kudlicki, W.; Odom, O. W.; Kramer, G.; Hardesty, B. *Biochemistry* **1993**, *32*, 7939−7945.
(8) The sequence of $tRNA_{NCCU}$(-CA) is pGCG GAU UUA GCU CAG UUG GGA GAG CGC CAG ACU NCCU AAU CUG GAG GUC CUG UGU UCG AUC CAC AGA AUU CGC AC.

(9) (a) Heckler, T. G.; Chang, L. -H.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1984**, *23*, 1468−1473. (b) Heckler, T. G.; Chang, L.-H.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *Tetrahedron* **1984**, *40*, 87−94.
(10) The reaction mixture contained 55mM Hepes−KOH (pH 7.5), 210 mM potassium glutamate, 6.9 mM ammonium acetate, 9 mM magnesium acetate, 1.7 mM dithiothreitol, 1.2 mM ATP, 0.28 mM GTP, 26 mM phosphoenolpyruvate, 1 mM spermidine, 1.9% polyethyleneglycol-8000, 35 μg/mL folinic acid, 0.1 mM each of amino acids except arginine, 0.01 mM arginine, 4 μg of mRNA, and 2 μL of *E. coli* S-30 extract in 10 μL. The tRNA charged with a nonnatural amino acid was added to 25 μM. The mixture was incubated at 37 °C for 60 min.

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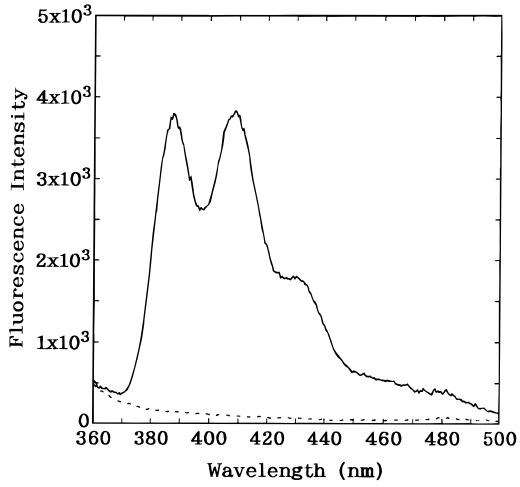

Figure 2. Fluorescence spectra of mutant streptavidin containing anthrylalanine (—), and wild type streptavidin (- - -) purified by Ni-NTA column (λ_{ex} = 256 nm).

2-anthryl groups were also successfully incorporated into streptavidin. From a dot blot analysis using biotinylated alkaliphosphatase, the yields of wild type and mutant streptavidin containing nitrophenylalanine were estimated to be 25 and, 5μg/mL, respectively. Thus, the suppression efficiency was around 20%.

Streptavidin synthesized with the $tRNA_{ACCU}$ charged with 2-anthrylalanine was purified by a Ni-NTA affinity column (QIAGEN). As shown in Figure 2, the streptavidin shows clear anthryl fluorescence, whereas a wild type protein that was mixed with anthrylalanine and purified through the same procedure, showed no anthryl fluorescence. The results indicate that anthrylalanine is covalently incorporated into streptavidin.

Other four-base codons, AGGG, AGGC, and AGGA, were also tested in the suppression system. As shown in Figure 3, all these codons are effective for the incorporation of nitrophenylalanine, when combined with the corresponding nitrophenylalanyl frame-shift suppressor tRNAs.

The frame-shift strategy is advantageous in extending the genetic code for the incorporation of nonnatural amino acids. In the previous system using a termination codon, only single

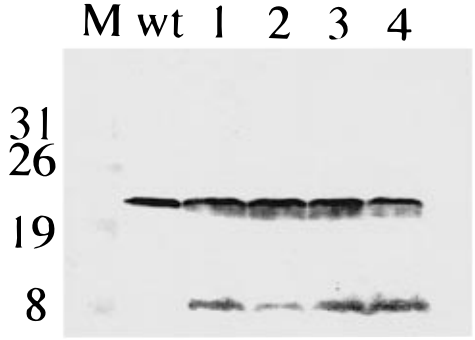

Figure 3. Western blot analysis of *in vitro* translation of mutant mRNA containing AGGN in the presence of nitrophenylalanyl $tRNA_{NCCU}$. The reaction contains the following: lane 1, mRNA with AGGU and $tRNA_{ACCU}$; lane 2, mRNA with AGGG and $tRNA_{CCCU}$; lane 3, mRNA with AGGC and $tRNA_{GCCU}$; lane 4, mRNA with AGGA and $tRNA_{UCCU}$.

nonnatural amino acid can be incorporated. The expansion of the genetic code has been also attempted by using *iso*-C and *iso*-G pair,[11] but the enzymatic transcription of the unusual base pair is a serious problem.

The present study shows that the limitation of the 64 codons can be overcome by the frame-shift strategy and opens a way to produce proteins that contain several different nonnatural amino acids at specific sites.

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Supporting Information Available: Preparation of aminoacyl dinucleotide and aminoacyl frame-shift suppressor tRNA (5 pages). See any current masthead page for ordering and Internet access instructions.

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(11) Bain, J. D.; Switzer, C.; Chamberlin, A. R.; Benner, S. A. *Nature* **1992**, *356*, 537−539.